

Purification of a Cortical Complex Containing Two Unconventional Actins from *Acanthamoeba* by Affinity Chromatography on Profilin-Agarose

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Abstract. We identified four polypeptides of 47, 44, 40, and 35 kD that bind to profilin-Sepharose and elute with high salt. When purified by conventional chromatography using an antibody to the 47-kD polypeptide, these four polypeptides copurified as a stoichiometric complex together with three additional polypeptides of 19, 18, and 13 kD that varied in their proportions to the other polypeptides. Partial protein sequences showed that the 47-kD polypeptide is a homologue of *S. pombe* *act2* and the 44-kD polypeptide is a homologue of *S. cerevisiae* *ACT2*, both unconventional actins. The 40-kD polypeptide contains a sequence similar to the WD40 motif of the G_β subunit of a trimeric G-protein from *Dictyostelium* *dis-*

coideum. From partial sequences, the 35-, 19-, and 18-kD polypeptides appear to be novel proteins. On gel filtration the complex of purified polypeptides cochromatograph with a Stokes' radius of 4.8 nm, a value consistent with a globular particle of 220 kD containing one copy of each polypeptide. Cell extracts also contain components of the complex that do not bind the profilin column. Affinity purified antibodies localize 47- and 18/19-kD polypeptides in the cortex and filopodia of *Acanthamoeba*. Antibodies to the 47-kD unconventional actin cross-react on immunoblots with polypeptides of similar size in *Dictyostelium*, rabbit muscle, and conventional preparations of rabbit muscle actin but do not react with actin.

PROFILIN was originally characterized as an actin monomer sequestering protein that forms a 1:1 complex with actin monomers in vitro and inhibits spontaneous nucleation of actin filaments (Carlsson et al., 1977; Pollard and Cooper, 1984; Lal and Korn, 1985). Profilin inhibits elongation more effectively at the pointed end of actin filaments than at the barbed end, since the actin profilin complex can bind to the barbed end but not the pointed end (Pollard and Cooper, 1984). Profilin also catalyzes actin nucleotide exchange by binding to actin monomers and lowering the affinity of actin for bound nucleotide (Mockrin and Korn, 1980; Goldschmidt-Clermont et al., 1991b). This activity may contribute to recycling ADP-actin released from depolymerizing filaments to ATP-actin ready for repolymerization during the turnover of filaments in the cell (Goldschmidt-Clermont et al., 1991b). Recent evidence suggests that profilin may even aid in the transfer of actin from the se-

questering protein, thymosin β 4 to the barbed end of actin filaments (Pantaloni and Carlier, 1993).

Profilin also binds to membrane phospholipids phosphatidylinositol 4 monophosphate (PIP)¹ and phosphatidylinositol 4,5 bisphosphate (PIP₂) and inhibits hydrolysis of these lipids by phospholipase C- γ (PLC- γ) (Goldschmidt-Clermont et al., 1990; Machesky et al., 1990). Phosphorylation of PLC- γ by the activated EGF receptor overcomes the profilin inhibition (Goldschmidt-Clermont et al., 1991a). This provides a plausible biochemical mechanism for regulation of PLC- γ by EGF and a possible way to release profilin from the membrane to interact with actin. Profilin does not bind to actin and phosphoinositides simultaneously (Lassing and Lindberg, 1985).

Poly-L-proline is another ligand of profilin (Tanaka and Shibata, 1985). This interaction has no known physiological function, but poly-L-proline Sepharose affinity columns have been very useful for purifying profilin (Kaiser et al., 1989; Janmey, 1991). It is conceivable that profilin binds to polypyrrolone sequences that are present in many cytoplasmic

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1. *Abbreviations used in this paper:* NaPPi, sodium pyrophosphate; PIP, phosphatidylinositol 4 monophosphate; PIP₂, phosphatidylinositol 4,5 bisphosphate; PLC- γ , phospholipase C- γ .

proteins, including calcineurin (Guerni and Klee, 1989), cyclase-associated protein (Field et al., 1990), vinculin (Coutu et al., 1987), and zyxin (Sadler et al., 1992).

In a search for new profilin ligands, we discovered seven proteins that bind to profilin-affinity columns. These seven polypeptides co-purify by conventional ion-exchange chromatography. Two of these proteins are the *Acanthamoeba* homologs of *S. cerevisiae* ACT2 and *S. Pombe* act2, two unconventional actins (or actin-related proteins). Several of these polypeptides are localized in the cortex of *Acanthamoeba* by immunofluorescence. Unconventional actins have been identified previously in yeast and vertebrates (reviewed by Herman, 1993). From their sequences, they are clearly homologues of actin, but they are less than 50% identical to conventional actins, all of which have highly conserved primary structures. The 47- and 44-kD unconventional actins were discovered during sequencing of cloned DNA from two species of yeast (Schwob and Martin, 1992; Lees-Miller et al., 1992b). The vertebrate unconventional actins of 46 kD are found as part of a complex associated with the microtubule motor dynein (Lees-Miller et al., 1992a) and concentrated near the centrosome (Clark and Meyer, 1992). Relatively little is known about the biochemical properties and functions of the unconventional actins.

Materials and Methods

Profilin-Sepharose Chromatography

A mixture of 30 mg profilin-I and profilin-II from *Acanthamoeba castellanii* (24 mg profilin-I, 6 mg profilin-II) was covalently linked to 10 g of CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) according to the manufacturer's instructions. This affinity resin was used in a 1.5 × 30 cm column at 4°C.

Acanthamoeba castellanii were grown in liquid culture, harvested by low-speed centrifugation and ~700 g cells were lysed in either pyrophosphate or sucrose buffer using a Parr nitrogen bomb (Korn et al., 1982). These buffers have been used for more than 15 yr to solubilize actin, myosin-I, myosin-II, and many of the cell's actin-binding proteins. Pyrophosphate buffer consisted of 150 mM KCl, 12 mM sodium pyrophosphate (NaPPi), 1 mM ATP, 0.1 mM benzamidine, 1 mM PMSF, 30 mM imidazole, pH 7.0, 5 mM dithiothreitol, 0.5 mM diisopropylfluorophosphate. Sucrose buffer contained 0.34 M sucrose, 1 mM EGTA, 1 mM dithiothreitol, 10 mM imidazole, pH 7.5, 1 mM ATP, 0.1 mM benzamidine, 1 mM PMSF. Homogenates were centrifuged at 100,000 g for 90 min at 4°C.

50 µl of a high-speed supernatant from a sucrose extract of *Acanthamoeba* were loaded onto either a 10 ml profilin-Sepharose 4B affinity column or a control column of 10 ml Sepharose 4B with either no protein or an irrelevant protein (a myosin II tail fragment) coupled. Columns were washed in 10 mM imidazole, pH 7.5, until no protein could be detected by the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA). The columns were eluted with a variety of different buffers as specified in the figure legends.

Protein Purification

Conventional Chromatography. Sucrose extracts of amoeba were chromatographed on a column of DEAE-cellulose equilibrated with 10 mM imidazole, pH 7.5, 0.5 mM ATP, 0.5 mM dithiothreitol, 0.2 mM CaCl₂, 0.1 mM benzamidine, 0.5 mM PMSF (Tseng et al., 1984). Pyrophosphate extracts were dialyzed into 7.5 mM NaPPi, 10 mM Tris-Cl, pH 8.0, 1 mM dithiothreitol, 0.6 mM PMSF before chromatography on the DEAE column equilibrated with 7.5 mM NaPPi, 10 mM Tris-Cl pH 8.0, 1 mM dithiothreitol, and 0.6 mM PMSF.

The 47-kD polypeptide was purified as follows by ion-exchange chromatography from the fractions that flowed through the DEAE column. In a sucrose preparation, potassium phosphate, pH 7.0, was added to a concentration of 20 mM. The DEAE flow-through was then applied to a 1.5 × 30

cm column (88 g) of hydroxylapatite (Bio-gel HTP, Bio-Rad Laboratories) equilibrated with 1 liter 50 mM potassium phosphate, pH 7.0, and washed with 200 ml 50 mM potassium phosphate, pH 7.0. This column was eluted with a 500 ml gradient of 50–400 mM potassium phosphate, pH 7.0. SDS-PAGE (Laemmli, 1970) and immunoblotting were used to detect the presence of the 47-kD polypeptide in fractions eluting at ~100 mM potassium phosphate. In the case of the pyrophosphate preparation, the peak fractions from the first hydroxylapatite column were pooled, dialyzed into 20 mM potassium phosphate, pH 7.0, and re-loaded onto a hydroxylapatite column (1.5 × 30 cm) equilibrated with 20 mM potassium phosphate, pH 7.0, and eluted with a 500 ml gradient of 50–400 mM potassium phosphate, pH 7.0. Peak fractions were detected by immunoblotting, pooled, and dialyzed into 20 mM KCl, 20 mM Tris-Cl, pH 7.5, 0.5 mM dithiothreitol, and loaded onto a 1.5 × 30 cm column of phosphocellulose (Whatman, Maidstone, England) pre-equilibrated with the dialysis buffer. This column was eluted with a 400 ml gradient of 20–500 mM KCl in 20 mM Tris-Cl, pH 7.5. The 47-kD polypeptide was identified by immunoblotting. As an additional step, we sometimes loaded peak fractions from the phosphocellulose column onto a 0.5 × 20 cm column of QAE-Sepharose (Bio-Rad Laboratories) equilibrated with 20 mM Tris-Cl, pH 7.5, 20 mM KCl, 0.5 mM dithiothreitol, washed with 100 mM NaCl, and eluted with 500 mM NaCl in 20 mM Tris-Cl, pH 7.5.

Poly-L-Proline Affinity Chromatography. The DEAE flow-through was fractionated by poly-L-proline Sepharose affinity chromatography (Kaiser et al., 1989). Proteins eluted with 4 M urea were dialyzed into 10 mM imidazole, pH 7.5, 20 mM NaCl. The proteins were chromatographed on the profilin-Sepharose affinity column and eluted with 0.4 M MgCl₂ column buffer. Profilin was eluted from the poly-L-proline column with 8 M urea (Kaiser et al., 1989).

Antibody Preparation and Purification

A New Zealand white rabbit (Bunnyville Farms, Littleton, PA) JH30 was immunized subcutaneously in four locations with 47-kD polypeptide purified by profilin-Sepharose chromatography and PAGE (Fig. 1, lane 12). The gel was stained for 1 h with 0.2% Coomassie blue, 0.1% SDS, 25 mM Tris base, 192 mM glycine, and destained for 10-min each in two changes of deionized H₂O. The gel slice was excised, frozen in liquid N₂, and pulverized to a fine powder with a mortar and pestle. The powder containing ~50 µg protein was added to 0.5 ml Freund's complete adjuvant and sonicated for 30 s with a probe sonicator (Branson, VWR, Bridgeport, NJ). This mixture was injected under the skin at 0 and 6 wk after a preimmune bleed. Antibodies from rabbit JH30 bound to one band on immunoblots of cell extracts (Fig. 2, lane A).

A polyclonal serum to the polypeptides that copurified with the 47-kD polypeptide, was prepared by immunizing rabbit JH43 with 500 µg of protein purified by ion-exchange chromatography (Fig. 3) in Freund's complete adjuvant boosting with antigen in Freund's incomplete adjuvant after 6 wk. Rabbit JH43 produced antibodies to most of the injected polypeptides (Fig. 2, lane B). From this complex serum we affinity-purified antibodies to the individual polypeptides (not attempting to separate the 19- and 18-kD antigens) using immunoblot strips (Pollard, 1984). Mock-purification of immune serum was carried out in an identical fashion with nitrocellulose strips from an immunoblot in a region containing no protein. Purified 47-kD antibody recognized one band at 47 kD on immunoblots of *Acanthamoeba* extracts (Fig. 2, lane C) and affinity purified 19/18-kD protein recognized one band (or a close-running doublet) (Fig. 2, lane D). Purified antibodies to 44-, 40-, 35-, and 13-kD polypeptides bound weakly to their antigens and had high backgrounds on immunoblots, so they had limited usefulness.

Antibodies to *Acanthamoeba* profilin were purified from previously characterized antisera of rabbits JH15 and JH16 (Tseng et al., 1984) using immunoblots containing recombinant *Acanthamoeba* profilin-I and profilin-II (Almo et al., 1994). Mouse monoclonal antibodies (4D6) that react with amoeba actin were obtained from Sigma Immunochemicals (St. Louis, MO) and Dr. James Lessard of the University of Cincinnati (Cincinnati, OH) (Lessard, 1988).

Crude extracts for immunoblotting were prepared from a variety of cells. Live *Dictyostelium discoideum* and *Acanthamoeba* were lysed in boiling SDS-PAGE sample buffer (10% wt/vol SDS, 1 M Tris-Cl, pH 6.8, 50% vol/vol glycerol, trace bromophenol blue, 5% vol/vol 2-mercaptoethanol). Conventionally purified actin (Spudich and Watt, 1971) was obtained from rabbit muscle acetone powder. Approximately equal protein loads as determined by the BCA assay (Pierce Biochemicals, Rockford, IL) were applied to SDS-PAGE, and blotted onto nitrocellulose. After reaction with antibodies, the blots were washed and bound antibodies detected with the ECL chemiluminescence system (Amersham Corp., Arlington Heights, IL).

Immunoblot Assay for Binding to Profilin Sepharose

10 g of packed cells were homogenized in 60 ml sucrose extraction buffer and centrifuged at 100,000 g for 90 min. 2 ml of the supernatant was applied to a 2 ml column of profilin-II-Sepharose. The column was washed with 100 ml of 10 mM imidazole, pH 7.5, 20 mM KCl, and eluted with 10 ml of 400 mM MgCl₂ in the same buffer. No other polypeptides were detected after elution with 8 M urea. Fractions were monitored for protein using a Bradford (1976) assay. Volumes of flow through and elution peaks were equivalent. Immunoblots using antiserum JH 43 were quantitated and used to calculate the fraction of each polypeptide present in the two fractions. Comparable results were obtained in two separate experiments using different extracts, and with both profilin-I- and profilin-II-Sepharose.

Gel Filtration

Stokes' radius was determined by the method of Siegel and Monty (1966) using a 1 × 120 cm column of Sephacryl S-300 equilibrated at 4°C with 75 mM KCl, 20 mM Tris-Cl, pH 7.5, and calibrated with Sigma gel-filtration standards (carbonic anhydrase, bovine serum albumin, β -amylase, and apoferritin). ATP marked the salt volume and blue dextran mol wt 2 million the void volume. Analytical gel-filtration was carried out at 22°C on a 0.5 × 50 cm column of Sephadex G-100 in 10 mM Tris-Cl, pH 7.5, 75 mM KCl, 0.5 mM dithiothreitol, with or without 5 M KSCN.

Immunofluorescence

Acanthamoeba castellanii were cultured in plastic dishes (Becton Dickinson & Co., Franklin Lakes, NJ) and processed for immunofluorescence (Yonemura and Pollard, 1992). Briefly, cells removed from plastic dishes by shaking were allowed to grow overnight on glass coverslips and fixed for 5 min in a 2% formaldehyde-methanol solution at -20°C. All subsequent steps were carried out at room temperature. Cells were washed in PBS (0.15 M NaCl, 0.01% NaN₃, 10 mM sodium phosphate, pH 7.4) and incubated with a 1:1 dilution of purified first antibody and a 1:50 dilution of rhodamine-labeled goat anti-rabbit (Organon Teknika, West Chester, PA).

Crude preimmune serum was used at a 1:500 dilution. Confocal fluorescence micrographs were taken with a Bio-Rad MRC 600.

Peptide Sequencing

The proteins in the profilin-binding complex were separated by SDS-PAGE on a large gel (Laemmli, 1970). Corresponding bands from several lanes were excised and applied to an elution and concentration gel (Vandekerckhove et al., 1993). The concentrated protein was blotted on Immobilon P membranes (Millipore) (Bauw et al., 1988), treated with trypsin, and the resulting peptides were separated by HPLC and run on an Applied Biosystems gas phase sequencer (model 470A) equipped with an on-line PTH amino acid analysis system (model 120A).

Quantitation

Gels and immunoblots were digitized and analyzed using Collage software (Fotodyne, New Berlin, WI) running on a Macintosh Quadra 650 computer.

Results

Several Polypeptides Bind to Profilin-Sepharose

The vast majority of polypeptides in soluble extracts of *Acanthamoeba* (Fig. 1, lane 1) flow through the affinity column with *Acanthamoeba* profilins coupled to agarose beads (Fig. 1, lanes 2–5). A 43-kD polypeptide, identified by microsequencing as conventional actin, bound weakly and eluted with low-salt buffer and buffer containing 5 mM ATP (Fig. 1, lanes 6–11). Polypeptides with molecular weights of 47, 44, 40, and 35 kD bound and were eluted with a high-salt

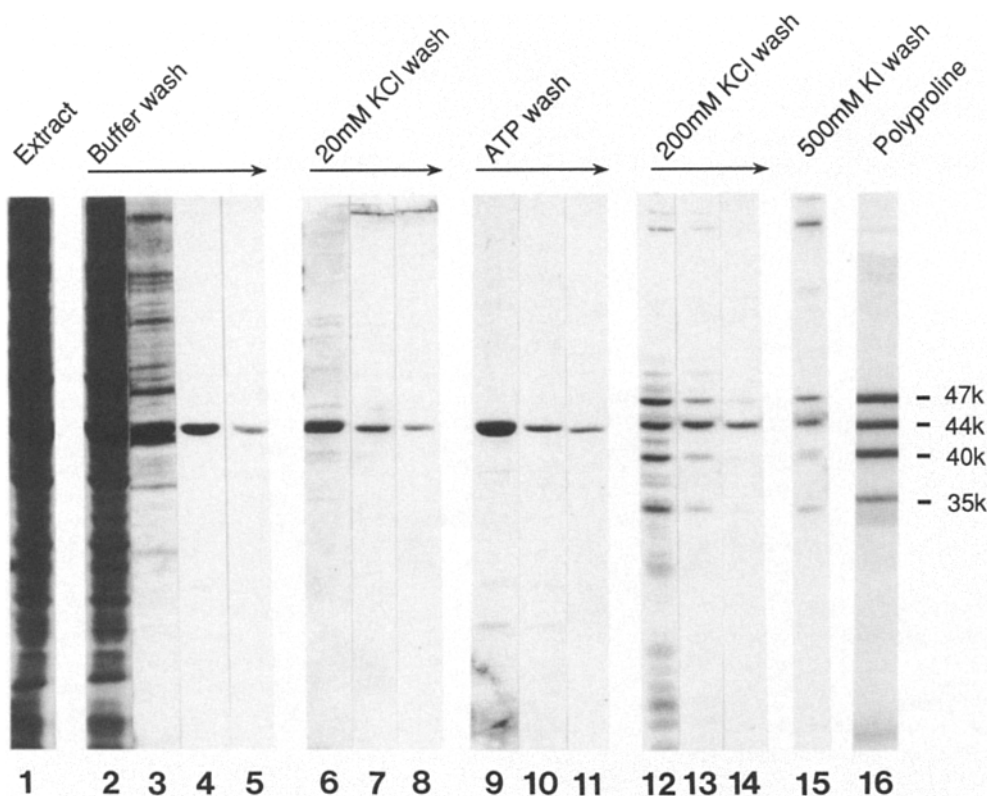


Figure 1. Affinity chromatography of *Acanthamoeba* extracts on a profilin-Sepharose column. Fractions were run on SDS-PAGE and stained with Coomassie blue. (Lane 1) A crude extract of *Acanthamoeba*. (Lanes 2–5) Flow through fractions. (Lanes 6–8) Proteins eluted with 10 mM, pH 7.5, imidazole, 20 mM KCl. (Lanes 9–11) Proteins eluted with 10 mM, pH 7.5, imidazole, 20 mM KCl, 5 mM ATP. (Lanes 12–14) Proteins eluted with 10 mM, pH 7.5, imidazole, 0.2 M KCl, 5 mM ATP. (Lane 15) Proteins eluted with 10 mM, pH 7.5, imidazole, 0.5 M KI. (Lane 16) Proteins purified by DEAE-cellulose, poly-L-proline, and profilin affinity chromatography. A crude *Acanthamoeba* extract was partially purified on DEAE cellulose. The flow through was adsorbed to poly-L-proline Sepharose, eluted with 4 M urea, renatured in low salt buffer, and then adsorbed to the profilin-agarose column. After washing with low-salt buffer, bound proteins were eluted with 10 mM Tris-Cl, pH 7.5, 0.4 M MgCl₂. Arrows indicate the positions of the 47-, 44-, 40-, 35-kD polypeptides.

buffer (Fig. 1, lanes 12–14). Anti-actin antibodies did not cross-react on immunoblots with the 44-kD species that eluted in high salt. KI eluted residual 47- and 44-kD polypeptide from the column (Fig. 1, lane 15). Several other polypeptides, including the low molecular weight polypeptides shown in Fig. 1 (lane 12) and a high molecular weight polypeptide shown in Fig. 1 (lanes 12 and 15) were present variably in fractions eluted by high salt. The Bradford assay detected no protein eluting from a control Sepharose 4B column without coupled protein. Polypeptides that eluted from a control column of a myosin-II tail fragment coupled to Sepharose did not correspond in molecular weight to those that eluted from profilin-Sepharose, nor did they react with antiserum JH43 (see below) on immunoblots. The experiment in Fig. 1 used a mixture of amoeba profilins immobilized on the agarose. Columns with either pure profilin-I or profilin-II gave similar results.

Polypeptides of 47, 44, 40, and 35 kD copurified with profilin during chromatography on DEAE-cellulose and affinity chromatography on poly-L-proline. These associated proteins were separated from profilin by elution of the poly-L-proline column with 4 M urea or 400 mM $MgCl_2$. (The profilin eluted from the poly-L-proline column with 8 M urea (Kaiser et al., 1989).) After removing the $MgCl_2$ or the urea by dialysis, the 47-, 44-, 40-, and 35-kD polypeptides rebound to the profilin affinity column (Fig. 1, lane 16). Binding of the complex to the profilin column was blocked by 30 μM profilin-I but not by 30 μM lysozyme. Fractions purified by poly-L-proline affinity chromatography contained variable amounts of polypeptides of ~19 and 13 kD.

Purification of the Profilin-binding Protein Complex by Ion Exchange Chromatography

A rabbit antiserum to the 47-kD polypeptide reacted specifically with the 47-kD polypeptide on immunoblots of amoeba extracts (Fig. 2, lane A) and with polypeptides of the same size from *Dictyostelium discoideum*, conventionally purified rabbit muscle actin and rabbit muscle extracts (not shown). However, neither the anti-47-kD antiserum nor purified anti-47-kD antibodies (Fig. 2, lane C) reacted with actin on immunoblots of pure amoeba or rabbit actin or actin in crude extracts (Fig. 2). Thus we could use these antibodies to assay for the 47-kD polypeptide during its purification by conventional chromatography.

We were surprised that polypeptides of 44, 40, and 35 kD (like those identified by profilin affinity chromatography) copurified with the 47-kD polypeptide through four ion exchange columns (Fig. 3). In addition, these fractions contained polypeptides of 19, 18, and 13 kD. Two different purification procedures produced the same complex of polypeptides.

We started the purification with a soluble extract, because an immunoblotting assay showed that the 47-kD polypeptide was found primarily in the soluble fraction of cells lysed in either sucrose or pyrophosphate. First extracts were chromatographed on DEAE-cellulose. About half of the 47-kD polypeptide flowed through with profilin. The other half was eluted with KCl along with actin (Tseng et al., 1984). The flow-through fractions contained profilin and the 47-kD protein as shown in Fig. 1 (lane 16). These fractions were chromatographed on hydroxylapatite and eluted with a linear gra-

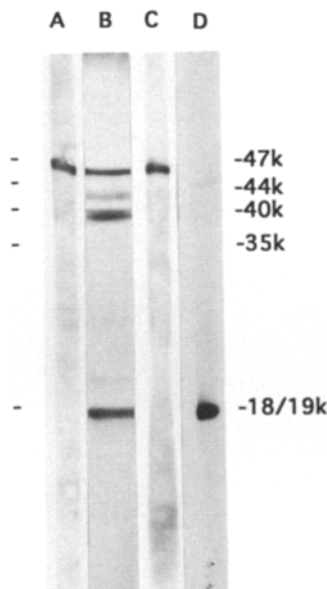


Figure 2. Immunoblots of a crude high-speed *Acanthamoeba* supernatant reacted with antisera and purified antibodies. (Lane A) Anti-47-kD immune serum from rabbit JH30. (Lane B) Antiserum to the profilin binding complex from rabbit JH43. (Lane C) JH43 affinity purified against 47 kD. (Lane D) JH43 affinity purified against 19/18 kD. Note that in lane B, the high concentration of conventional actin in the crude extract competes with 44K for binding to the nitrocellulose, reducing the signal from 44K on this immunoblot compared with the more purified fraction shown in Fig. 5.

dient of 20–400 mM potassium phosphate. The 47-kD polypeptide eluted in 150 mM potassium phosphate. (Peak fractions from the pyrophosphate preparation were dialyzed into 20 mM potassium phosphate, pH 7.0, and rerun on a second hydroxylapatite column.) Peak fractions from hydroxylapatite were run on phosphocellulose and eluted with a 20–500 mM linear KCl gradient. The 47-kD protein copurified with polypeptides of 44, 40, 35, 19, 18, and 13 kD (Fig. 3 A). Further chromatography of a pyrophosphate extract on QAE-Sepharose did not separate these seven polypeptides (Fig. 3 B). Copurification of these seven polypeptides through several ion-exchange columns suggested that they might form a complex that interacts with profilin. Polypeptides purified in this way bound to profilin Sepharose and were eluted with buffer containing 0.4 M $MgCl_2$.

The yield of the seven polypeptides was about 1 mg from ~700 g packed *Acanthamoeba*, from either sucrose or

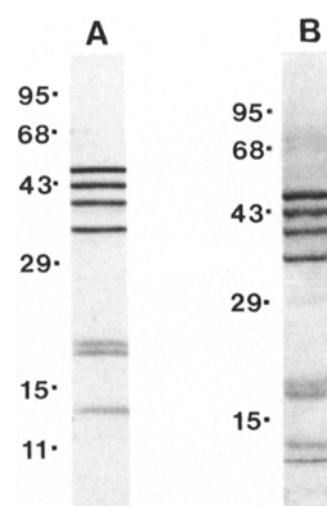


Figure 3. SDS-PAGE and Coomassie blue stain of two separate preparations of the profilin binding complex purified by ion-exchange chromatography using anti-47-kD antiserum to assay fractions. (Lane A) Polypeptides purified using sucrose lysis buffer and chromatography on DEAE-cellulose, hydroxylapatite, and phosphocellulose. The molar ratios of the 44-, 40-, 35-, 19-, 18-, and 13-kD polypeptides relative to 47 kD were 1.0, 0.8, 0.8, 0.4, 0.4, and 0.3, respectively. (Lane B) Polypeptides purified using pyrophosphate lysis buffer and chromatography on DEAE-cellulose, hydroxylapatite, phosphocellulose, and QAE-Sepharose. The molar ratios of the 44-, 40-, 35-, 19-, 18-, and 13-kD polypeptides relative to 47 kD were 1.1, 1.0, 1.1, 0.8, 2.1, and 0.6, respectively.

pyrophosphate preparations. This is considerably less than routine yields of profilin (100 mg) or actin (>200 mg) from these extracts.

Physical Properties of the Profilin-binding Complex

The seven purified polypeptides eluted together as a single peak with a Stokes' radius of 4.8 nm during analytical gel filtration. A spherical particle with a partial specific volume of 0.73 and this Stokes' radius would have a molecular weight of 220 kD. Gel filtration in 5 M KSCN partially dissociated the 47-, 44-, 40-, and 35-kD polypeptides from each other and completely separated the 19/18- and 13-kD polypeptides from the higher molecular weight polypeptides.

The 47- and 44-kD Polypeptides Are Unconventional Actins

By peptide sequencing of bands from Coomassie blue stained gels, we identified two of the copurifying polypeptides as actin-related proteins (Fig. 4). The sequence of the

47-kD protein is similar to the predicted amino acid sequence of *C. elegans* act d (Waterston et al., 1992), bovine act2 (Tanaka et al., 1992), and *S. pombe* act2 (Lees-Miller et al., 1992b). Peptides from the 44-kD protein are similar to the predicted amino acid sequence of *C. elegans* act c (Waterston et al., 1992) and *S. cerevisiae* ACT2 (Schwob and Martin, 1992). The 44-kD polypeptide did not react with actin antibodies on immunoblots and no actin peptides were detected in the 44-kD band by microsequencing. The identity of both amoeba proteins has been confirmed by sequencing full length cDNAs (Kelleher, J., S. Atkinson, and T. D. Pollard, unpublished results).

The 40-kD Polypeptide Is Similar to the G_{β} Subunit of a Trimeric G Protein

A 10-amino acid polypeptide fragment of the 40-kD protein (Fig. 4, peptide p1) contains 5 amino acids which are identical to a WD40 motif (Iniguez-Lluhi et al., 1993) found in the 40-kD G_β subunit of a *Dictyostelium* G protein (Pupillo et al., 1988). This G protein β -subunit is essential for aggregation of cells during the early stages of *Dictyostelium* development (Lilly et al., 1993). Further sequence data will be required to confirm the identity of this amoeba protein.

The partial peptide sequences of the 35-, 19-, and 18-kD polypeptides are not similar to any proteins in the PIR database (Fig. 4). We were unable to obtain sequences of peptides from the 13-kD protein. Antibodies to amoeba profilin did not react with the 13-kD polypeptide or any of the other polypeptides in the complex.

Stoichiometry of the Polypeptides in the Profilin-binding Complex

Assuming equal dye binding, the 47-, 44-, 40-, and 35-kD polypeptides were present in equal amounts in the purified complex (Fig. 3, legend). The 19-, 18-, and 13-kD components were substoichiometric and differed in the two preparations that we quantitated (Fig. 3).

We do not know the stoichiometry of the seven polypeptides in the cell, but fractionation on the profilin-affinity column suggests that some of these polypeptides are present in excess in the cell extract. First, when limiting amounts of extract are adsorbed to the profilin column, much of each of the polypeptides in the purified complex binds to the column, but different proportions of each flow through the column (Fig. 5). Nearly all of the 40-, 35-, 19-, and 18-kD components bound to the column, but 35% of 47 kD and 20% of 44 kD are unbound. Second, during the high-salt elution of the polypeptides bound to the profilin column, part of both unconventional actins trail behind the main peak of complex and are eluted by KI (Fig. 1, lanes 12-15).

The 47- and 19/18-kD Polypeptides Are Localized in the Cortex of Acanthamoeba

Three members of the purified complex localize to filopodia and cortex of *Acanthamoeba* along with actin filaments and some of the profilin. Affinity-purified antibodies to 47-kD unconventional actin stained filopodia and the cortex of *Acanthamoeba* (Figs. 6 and 7 B) strongly compared with the rest of the cytoplasm. By confocal microscopy 47-kD unconventional actin was concentrated in spots corresponding to filopodia on the free surface of cells (Fig. 6 A) and through-

37k unconventional actin						19									32
Acanthamoeba actin	A	G	F	A	G	D	D	A	P	R	A	V	F	P	
Acanthamoeba 47k p17b	M	G	I	Y	A	G	N	T	E/L	P	Q	Y/F	I	P	
S. elegans act d	L	G	Y	A	G	N	T	E	P	Q	F	I	I	P	
S. pombe act 2	L	G	Y	A	G	N	D	A	P	S	Y	V	F	P	
Bovine act 2	L	G	Y	A	G	N	T	E	P	Q	F	I	I	P	
						46									63
Acanthamoeba actin	G	M	G	Q	K	D	S	Y	V	G	D	E	A	Q	S K R G
Acanthamoeba 47k p22	N	I	A	D	L	D	F	F	I	G	D	E	A	Y	E N S K
C. elegans act d	G	I	D	D	L	D	F	F	I	G	D	E	A	L	S P A A
S. pombe act 2	A	T	E	D	L	D	F	F	I	G	N	D	A	L	K C A S
Bovine act 2	G	V	D	D	L	D	F	F	I	G	D	E	A	I	E K P Y
						147									161
Acanthamoeba actin	R	T	T	G	I	V	L	D	S	G	D	G	V	T	H
Acanthamoeba 47k p21	X	L	T	G	T	V	I	D	S	G	D	G	V	T	X
C. elegans act d	S	L	T	G	L	V	I	D	S	G	D	G	V	T	H
S. pombe act 2	S	L	T	G	T	V	V	D	S	G	D	G	V	T	H
Bovine act 2	T	L	T	G	T	V	I	D	S	G	D	G	V	T	H
						339									350
Acanthamoeba actin	N	V	V	L	S	G	G	T	T	M	F	P			
Acanthamoeba 47k p17a	N	I/G	V	L	S	G	G	L/E	T	M	F/Y	K			
C. elegans act d	N	I	V	L	S	G	G	S	T	M	Y	K			
S. pombe act 2	N	I	V	L	S	G	G	S	T	L	F	K			
Bovine act 2	N	I	V	L	S	G	G	S	T	M	F	R			
44k unconventional actin						63									76
Acanthamoeba actin	G	I	L	T	L	K	Y	P	I	E	H	G	I	V	
Acanthamoeba 44k p20	X	M	L	Q	I	T	Y	P	L	D	N	G	I	V	
C. elegans act c	Q	M	L	D	I	N	Y	P	M	D	N	G	I	V	
S. cerevisiae ACT 2	S	Y	L	Q	I	S	Y	P	M	E	N	G	I	I	
						78									94
Acanthamoeba actin	N	W	D	D	M	E	K	I	W	H	H	T	F	Y	N E L
Acanthamoeba 44k p21	N	W	E	D	A	E	H	V	W	N	Y	T	F	F	E K
C. elegans act c	N	W	D	D	M	E	H	V	W	D	H	T	F	G	P E K
S. cerevisiae ACT 2	N	W	T	D	M	E	L	L	W	D	Y	A	F	F	E Q M
40k polypeptide															
Acanthamoeba 40k p1	L	V	W	D	V	A	S	L	V	K					
Dictyostelium G-beta 240-249	L	V	W	D	G	L	T	T	N	K					
Dictyostelium G-beta 531-540	I	L	W	D	V	E	N	G	T	K					
Mus I G-beta WD 336-345	I	I	W	D	S	Y	T	T	N	K					
Acanthamoeba 40k p2	X	K	P	T	L	V	I	L	R						
Acanthamoeba 40k P3	V	F	S	A	X	V	K								
18, 19 & 35k polypeptides															
Acanthamoeba 35k p1	V	F	L	Q	E	L	E	A	A	R					
Acanthamoeba19k p1	I	V	A	G	S	Y	L	K							
Acanthamoeba19k p2	X	X	N	F	V	V	L	R							
Acanthamoeba18k p1	Y	T	G	P	A	D	Q	P	D	I	I	D	E	A	I X F

Figure 4. Partial amino acid sequences of the 47-, 44-, 40-, 35-, 19-, and 18-kD polypeptides. These sequences are aligned with homologous sequences of conventional *Acanthamoeba* actin and other unconventional actins and the G_{β} subunit of a trimeric G protein. References: *Acanthamoeba* conventional actin (Nellen and Gallwitz, 1982); *C. elegans* act c and act d (Waterston et al., 1992); *S. cerevisiae* ACT2 (Schwob and Martin, 1992); *S. pombe* act2 (Lees-Miller et al., 1992b); *Dictyostelium*; and mouse G_{β} subunits (Lilly et al., 1993).

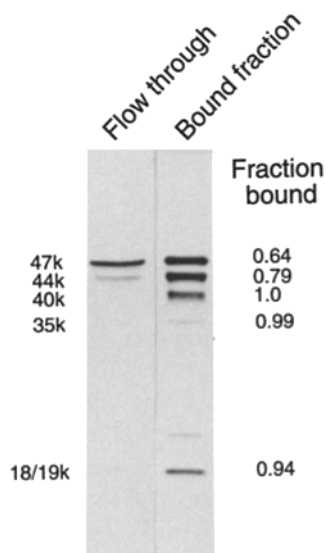


Figure 5. Immunoblot assay for binding of *Acanthamoeba* polypeptides to a profilin-Sepharose affinity column. Immunoblot using antiserum JH43 of the flow-through fraction (lane 1) and the fraction that eluted with 0.4 M MgCl_2 (lane 2). The fraction of each polypeptide bound to the column is indicated. Note that the polyspecific antiserum used for this experiment is useful for comparing the amount of each component in the bound and free fractions, but was not useful for quantitating the stoichiometry within the complex, since the reaction with 35 kD is weak compared to the other bands.

out the cortex at all levels of the cell (Fig. 6, B and D). Purified antibodies to 44-kD unconventional actin also stain the cortex and filopodia (J. Kelleher, unpublished results).

Antibodies to the 19- and 18-kD polypeptides stained filopodia and the cortex of *Acanthamoeba* (Fig. 7 D) strongly compared with the rest of the cytoplasm. Purified 19/18-kD antibodies reacted only with polypeptides of this size on immunoblots of amoeba extracts (Fig. 2, lane D). Mock-purified serum stained the cytoplasm and contents of vacuoles weakly (Fig. 7 F). Affinity-purified antibodies to the 44-, 40-, and 35-kD polypeptides also stained the cortex, but these antibodies cross-reacted with the 47-kD polypeptide on immunoblots, so they are not illustrated.

Profilin and actin are concentrated in the cortex (Fig. 8, A-I) along with the 47-kD unconventional actin and 19/18-kD polypeptides. Affinity-purified polyclonal antibodies to *Acanthamoeba* profilin-I and profilin-II stain the cytoplasm as observed previously (Tseng et al., 1984) but they also stain striking cortical patches. Fibroblasts also have cortical patches of profilin (Buss et al., 1992). Purified antibodies from the sera of rabbits JH34 and JH35 gave the same result. Preimmune serum did not stain the cells at a dilution of 1:500. A monoclonal antibody to actin (Fig. 7, F-I) and rhodamine-phalloidin also stained the cortex and filopodia. By confocal microscopy this staining was more uniform than that with the anti-47-kD unconventional actin.

Discussion

Two unconventional actins with several other polypeptides from crude extracts of *Acanthamoeba* bind to profilin-agarose and copurify by ion-exchange chromatography. At least three of the seven are localized together in the cortex of *Acanthamoeba*. The unconventional actins are homologues of *S. pombe* *act2* and *S. cerevisiae* *ACT2*. We present the first biochemical purification and initial characterization of members of these families of proteins. The evidence suggests that these unconventional actins interact with proteins of the actin cytoskeleton, specifically with profilin and conventional actin (given the presence of 47 kD in actin purified from rabbit muscle). Given these associations and their es-

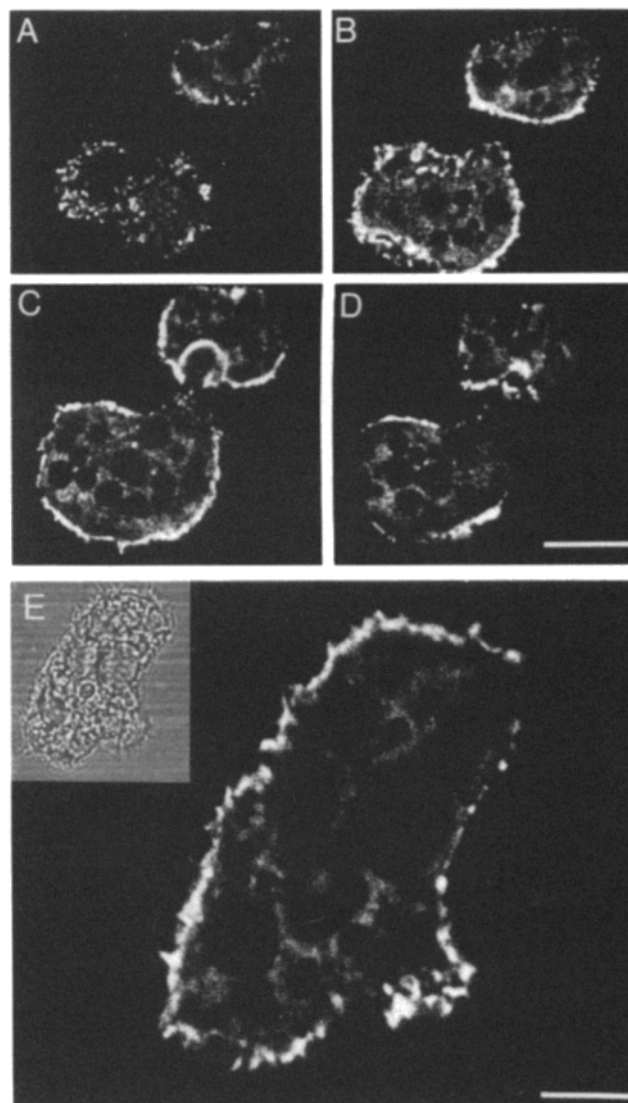


Figure 6. Localization of the 47-kD unconventional actin in *Acanthamoeba* by indirect immunofluorescence with affinity-purified antibodies. Confocal sections at 2- μm intervals from (A) the top of the cell showing many filopodia to (D) the bottom surface next to coverslip. The cortex is stained throughout. (E) Confocal fluorescence micrograph of another *Acanthamoeba*. The inset is a phase contrast micrograph. Bars: (A-D) 10 μm ; (E) 5 μm .

sential nature in yeast, we anticipate an exciting role for these proteins in the cell.

Since amoeba is closer to the root of the eukaryotic phylogenetic tree than yeast or vertebrates (Olsen and Woese, 1993), the presence of the 47 and 44 kD unconventional actins in amoeba confirms that these proteins are ancient and likely to be present in many eukaryotes. The sequences of these two unconventional actins from yeast, amoeba and vertebrates are remarkably similar. They clearly stand apart from each other and conventional actins, which have at least 70% amino acid identity from protozoa to plants, fungi, and animals. The 44-kD unconventional *ACT2* from *S. cerevisiae* is only 47% identical to yeast actin (Schwob and Martin, 1992), but much more similar to its homologues from *Dictyostelium* (S. Atkinson, manuscript in

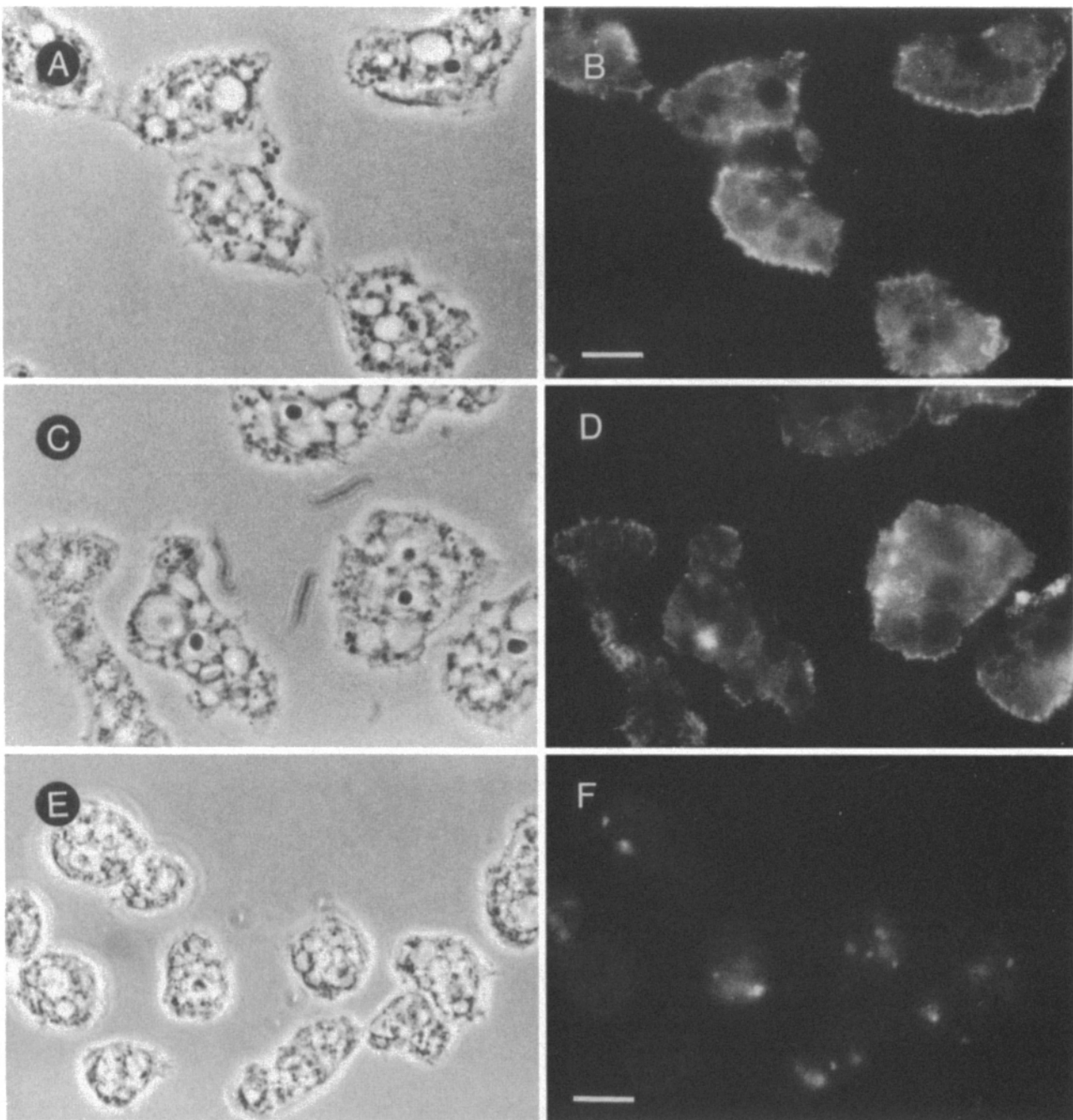


Figure 7. Comparison of the localization of the 47- and 18/19-kD polypeptides by indirect immunofluorescence with affinity purified antibodies. (A, C, and E) Phase contrast micrographs. (B) Fluorescence micrograph with anti-47kD. (D) Anti-19/18kD. (F) Control fluorescence micrograph with mock-purified immune serum. Bar, 10 μ m.

preparation) and *Acanthamoeba*. The 47-kD unconventional *act2* from *S. pombe* is only 35–40% identical to conventional actins, including its own *act1*, but has similar 47-kD homologues in cows (Tanaka et al., 1992), *Dictyostelium* and *Acanthamoeba*.

Properties of the Profilin-binding Complex

The copurification of seven polypeptides through five conventional chromatography columns indicates that they form

a relatively stable complex. The Stokes' radius of the purified material is consistent with a globular complex containing one copy of each of the seven polypeptides, but more rigorous studies are required to establish the stoichiometry definitively. Some of the components of the complex, particularly the 47- and 44-kD unconventional actins, appear to be in excess in cell extracts, so in the cell some of the seven polypeptides must be free or have associations outside of the complex.

Unconventional actins are the best candidates in the complex for binding profilin. Since the complex bound to the

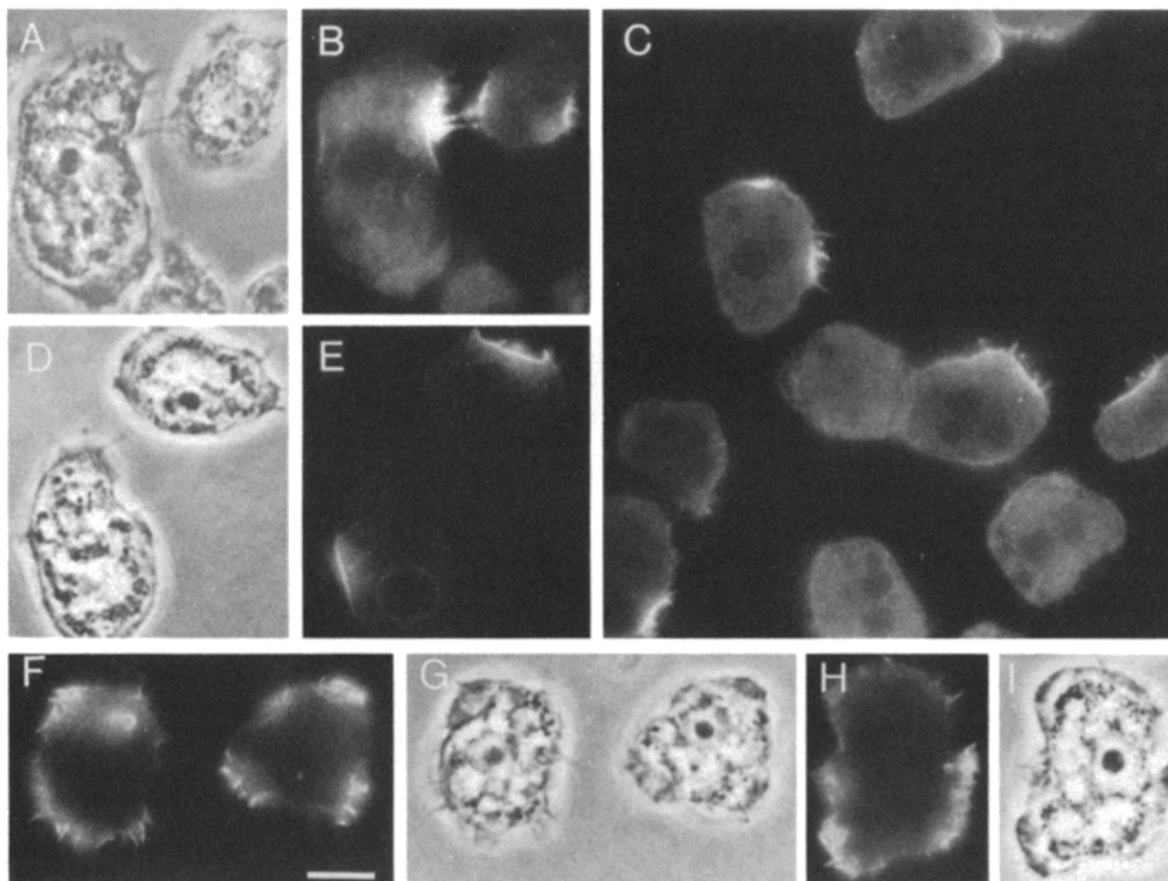


Figure 8. Localization of profilin and actin in *Acanthamoeba* by indirect immunofluorescence with affinity purified antibodies. (A, D, G, and H) Phase contrast micrographs. (B, C, and E) Fluorescence micrograph with anti-profilin. (F and H) Fluorescence micrograph with anti-actin. Bar, 10 μ m.

profilin-affinity column more tightly than actin (Fig. 1), one or both of the unconventional actins may have a higher affinity for profilin than conventional actin. This will be tested once the individual components are purified in quantities adequate for binding studies.

Compared with conventional actin, the components of the complex are minor cellular proteins judging from affinity chromatography of cellular extracts on profilin-Sepharose (Fig. 1). Bovine *act2* is also predicted to be a minor protein (Tanaka et al., 1992). Clearly these proteins have a different role than conventional actins.

Functions of Unconventional Actins

To date only the actin-RPV/contractin class of unconventional actins has been studied biochemically. They represent a third class of unconventional actins, different from the two that we have isolated. These 46-kD unconventional actins are reported to be concentrated in centrosomes and perinuclear regions of MDCK cells (Clark and Meyer, 1992). They are also part of the dynactin complex (Lees-Miller et al., 1992a), which co-purifies with cytoplasmic dynein (Paschal et al., 1993) and promotes dynein based motility of vesicles along microtubules (Gill et al., 1991; Schroer and Sheetz, 1991).

Our work establishes that two other unconventional actins,

the 47-kD homologue of *S. cerevisiae* *ACT2* and the 44-kD homologue of *S. pombe* *act2*, are present in low concentrations in the cortex of amoebas, apparently in association with each other and a small number of other polypeptides. The low abundance of the complex and its association with profilin and actin in the cortex of the cell, suggest to us that the complex has a regulatory rather than a structural role in the cell. This suggestion is consistent with genetic studies in yeast. Overexpression of the budding yeast *ACT2* gene for the 44-kD unconventional actin results in large rounded cells that do not bud (Schwob and Martin, 1992). Deletion of this gene is lethal late in cytokinesis of budded cells. Deletion of the 47-kD unconventional *act2* gene from fission yeast is also lethal (Lees-Miller, et al., 1992b).

Further work is required to learn whether the unconventional actin complex from the amoeba is regulated by profilin or regulates one of the known functions of profilin such as its interactions with actin or the phosphoinositide signaling pathway. Another possibility is that the unconventional actins in the complex dimerize to form a cryptic nucleus for actin filament formation or an actin filament cap. The 40-kD polypeptide or other components in the complex could regulate these activities.

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